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<b>(21) International Application Number:</b> PCT/US89/05027 <b>(22) International Filing Date:</b> 9 November 1989 (09.11.89) <b>(30) Priority data:</b> 269,945                      10 November 1988 (10.11.88) US 307,817                      7 February 1989 (07.02.89) US <b>(71) Applicants:</b> GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). THE WISTAR INSTITUTE [US/US]; 36th and Spruce Streets, Philadelphia, PA 19104 (US). <b>(72) Inventors:</b> TRINCHIERI, Giorgio ; 355 Wister Road, Wynnewood, PA 19104 (US). PERUSSIA, Bice ; 2302 Waverly Street, Philadelphia, PA 19146 (US). KOBAY- ASHI, Michiko ; B-513 Ookurayama, 931-1 Mametocho Kokoku-ku, Kanagawa, Yokohama (JP). CLARK, Ste- ven, C. ; 122 Johnson Road, Winchester, MA 01890 (US). WONG, Gordon, G. ; 40 Jamaica Way, Apartment 10,		<b>Jamaica Plain, MA 02130 (US). HEWICK, Rodney, M. ; 16</b> <b>Woodcliffe Road, Lexington, MA 02173 (US).</b> <b>(74) Agent:</b> KAPINOS, Ellen, J.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US). <b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CH (European patent), DE (European pa- tent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>

**(54) Title:** NATURAL KILLER STIMULATORY FACTOR**(57) Abstract**

A novel homogeneous human cytokine, Natural Killer Stimulatory Factor, and pharmaceutical preparation containing it.

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NATURAL KILLER STIMULATORY FACTOR

This is a continuation-in-part of pending U. S. patent application Serial Number 07/269,945, filed November 10, 1988.

The present invention relates to a novel cytokine that stimulates the function of natural killer cells and other cells of the immune system, and to processes for obtaining the factor in homogeneous form and producing it by recombinant genetic engineering techniques.

Background of the Invention

Natural killer (NK) cells are a subset of lymphocytes active in the immune system and representing an average 15% of mononuclear cells in human peripheral blood [G. Trinchieri and B. Perussia, Lab. Invest., 50:489 (1984)]. Among the surface markers used to identify human NK cells is a receptor binding with low affinity to the Fc fragment of IgG antibodies, such as Fc-gamma receptor III or CD16 antigen [B. Perussia et al, J. Immunol., 133:180 (1984)]. NK cells have been demonstrated to play an important role in vivo in the defense against tumors, tumor metastases, virus infection, and to regulate normal and malignant hematopoiesis.

A growing family of regulatory proteins that deliver signals between cells of the immune system has been identified. These regulatory molecules are known as cytokines. Many of the cytokines have been found to control the growth, development and biological activities of cells of the hematopoietic and immune systems. These regulatory molecules include all of the colony-stimulating factors (GM-CSF, G-CSF, M-CSF, and multi CSF or interleukin-3), the interleukins (IL-1 through IL-7), the interferons (alpha, beta and gamma), the tumor necrosis factors (alpha and beta) and leukemia inhibitory factor (LIF). These cytokines exhibit a wide range of biologic activities with target cells from bone marrow, peripheral blood, fetal liver, and other lymphoid or hematopoietic organs. See, e.g., G. Wong and S. Clark, Immunology Today, 9(5):137 (1988).

The biochemical and biological identification and characterization of certain cytokines was hampered by the small quantities of the naturally occurring factors available from natural sources, e.g., blood and urine. Many of the cytokines have recently been molecularly cloned, heterologously expressed and purified to homogeneity. [D. Metcalf, "The Molecular Biology and Functions of the Granulocyte-Macrophage Colony Stimulating Factors," Blood, 67(2):257-267 (1986).]

Among these cytokines are gamma interferon, human and murine GM-CSF, human G-CSF, human CSF-1 and human and murine IL-3. Several of these purified factors have been found to demonstrate regulatory effects on the hematopoietic and immune systems in vivo, including GM-CSF, G-CSF, IL-3 and IL-2.

There remains a need in the art for additional proteins purified from their natural sources or otherwise produced in homogeneous form, which are capable of stimulating or enhancing immune responsiveness and are suitable for pharmaceutical use.

#### Brief Summary of the Invention

In one aspect the present invention provides a novel human natural killer stimulatory factor, called NKSF, which is substantially free from other mammalian proteins. Active NKSF has an apparent molecular weight of approximately 70 kd. Pure preparations of NKSF reveal the presence of two polypeptides, which are contemplated as subunits which, when associated, yield active NKSF. It is presently speculated that NKSF is a heterodimer formed by association of both the larger and smaller subunits through one or more disulfide bonds. This apparent heterodimeric structure could be generated by association of the two individual subunits or by proteolytic cleavage of a single precursor polypeptide,

e.g. as with insulin. Alternatively, it is possible that the active form of NKSF is a homodimer of the larger subunit or a homodimer of the smaller subunit.

The active approximately 70-80 kd NKSF is further characterized by containing all or a portion of the amino acid sequence of Table I below. Additionally, one or more of the following sequences of amino acids (in single letter code) is present in the primary sequence of either the larger or smaller of the NKSF subunits.

( ) indicate an amino acid not positively identified.

L-T-I-Q-V

K-Y-E-N-Y-T

I-W-E-L-K

L-M-D-P-K

V-M-S-Y-L-N-A

(A)-V-S-N-M-L-Q-K

N-A-S-I-S-V

T-F-L-R

D-I-I-K-P-D-P-P-K.

The larger subunit polypeptide of NKSF is characterized by having an apparent molecular weight of 40kd. This subunit is further characterized by having the same or substantially the same amino terminal sequence as follows:

I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-G-E-M.

This larger polypeptide is further characterized by containing all or a portion of the longer cloned sequence as disclosed in Table I below.

The smaller polypeptide subunit of NKSF is characterized by an apparent molecular weight of approximately 30-35 kd and is further characterized by having the same or substantially the same amino terminal sequence as follows:

(X)-N-L-P-V-A-(P)-P-D-P-(S or T)-M-F-P. The (X) indicates that the first residue of the sequence of the smaller subunit could not be determined.

NKSF displays biological activity in inducing the production of gamma interferon in vitro by human peripheral blood lymphocytes (PBLs). In homogeneous form, NKSF is characterized by a specific activity of greater than  $1 \times 10^7$  dilution units per milligram in the gamma interferon induction assay, described in detail below.

In addition to the induction of gamma interferon in PBLs, NKSF demonstrates the following biological activities:

(1) biological activity in a granulocyte-macrophage colony stimulating factor (GM-CSF) inducing assay with PBLs;

(2) biological activity in activating Natural Killer (NK) cells to kill leukemia and tumor-derived cells;

(3) biological activity in a tumor necrosis factor (TNF) inducing assay with phytohemagglutinin (PHA)-activated T lymphocytes; and

(4) co-mitogenic activity with peripheral blood T lymphocytes.

Another aspect of the invention includes DNA sequences comprising cDNA sequences coding on expression for a human NKSF polypeptide, a human NKSF larger subunit polypeptide, and a human NKSF smaller subunit polypeptide. Such sequences include a sequence of nucleotides encoding one or more of the subunits and peptide sequences described above.

Also provided by the present invention is a vector containing a DNA sequence encoding NKSF or a subunit of NKSF in operative association with an expression control sequence. Host cells transformed with such vectors for use in producing recombinant NKSF or its recombinant subunits are also provided by the present invention.

As still a further aspect of the present invention, there is provided recombinant NKSF protein. This protein is free from other mammalian proteinaceous



materials and is characterized by the presence of a DNA sequence encoding one or more of the above-described subunits or peptide fragments containing one or more of the above-described physical, biochemical or biological activities or characteristics.

Another aspect of this invention provides pharmaceutical compositions containing a therapeutically effective amount of homogeneous or recombinant NKSF, or an effective amount of one or both of the subunits of NKSF, or of one or more of the peptide fragments thereof. These pharmaceutical compositions may be employed in methods for treating cancer, and other disease states responsive to the enhanced presence of gamma interferon and GM-CSF production. Thus, generally this factor may be employed in the treatment of diseases characterized by a deficiency in the number or level of activity of hematopoietic cells.

A further aspect of the invention, therefore, is a method for treating cancer and/or other pathological states which may benefit from enhanced natural killer cell functions by administering to a patient a therapeutically effective amount of NKSF or one or both of its subunits or peptide fragments thereof in a suitable pharmaceutical carrier. These therapeutic methods may include administering simultaneously or

sequentially with NKSF or one or more of its subunits or peptide fragments an effective amount of at least one other cytokine, hematopoietin, interleukin, growth factor, or antibody.

5           Still a further aspect of the present invention is a process for producing homogeneous NKSF, or a subunit thereof from a human cell line producing NKSF or a subunit thereof in admixture with other proteins and polypeptides. This process of production provided by the  
10           present invention includes culturing selected cells capable of producing NKSF, its subunits, or peptide fragments thereof to obtain conditioned medium and purifying the conditioned medium through five primary purification steps.

15           The vectors and transformed cells of the invention are employed in another aspect, a novel process for producing recombinant human NKSF protein, a subunit thereof or peptide fragments thereof. In this process a cell line transformed with a DNA sequence encoding on  
20           expression NKSF protein, a subunit thereof or a peptide fragment thereof in operative association with an expression control sequence therefore is cultured. This claimed process may employ a number of known cells as host cells for expression of the polypeptide. Presently

preferred cell lines are mammalian cell lines and bacterial cells.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description of preferred embodiments thereof.

#### Detailed Description of the Invention

The novel human natural killer cell stimulatory factor, NKSF, provided by the present invention is a homogeneous protein or proteinaceous composition substantially free of association with other mammalian proteinaceous materials.

Natural killer stimulatory factor has an apparent molecular weight of approximately 70-80 kd as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. This 70-80 kd peptide is active in a gamma interferon induction assay.

Under reducing conditions in SDS-PAGE, the 70-80 kd band yields two smaller subunits with apparent molecular weights of 40 kd (larger subunit) and approximately 30-35 kd (smaller subunit). For both subunits, the biological activity in the same gamma interferon induction assay is substantially lost compared to that of the native 70-80 kd species. The amino

terminal sequences identified above were determined from the 40 kd reduced species and the 30-35 kd reduced species believed to be the subunits of the NKSF heterodimer. It is presently believed that NKSF is a disulfide-bonded heterodimer of the larger and smaller subunits.

NKSF is, at least in part, an anionic glycoprotein. Under isoelectric focusing, two species of the NKSF are observed having isoelectric points of 4.3 and 4.8. It is presently speculated that the two species differ in glycosylation patterns.

NKSF is primarily characterized by biological activity in the gamma interferon induction assay described in detail in Example 8 below. Among its other biological activities include the ability to induce GM-CSF production by human peripheral blood lymphocytes. [See, e.g., published PCT application WO86/00639 for additional information on GM-CSF]. NKSF also has an enhancing effect on the mitogenic activity of various mitogens, such as lectins and phorbol diesters, on peripheral blood T lymphocytes and has a growth promoting effect on activated human tonsillar B cells.

NKSF has also been observed to enhance NK cell functions to kill leukemia and tumor-derived cells in

vitro using a spontaneous cell cytotoxicity assay and an antibody dependent cell cytotoxicity (ADCC) assay.

Briefly in a spontaneous cell cytotoxicity assay, human peripheral blood lymphocytes or purified NK cells are incubated in the presence of NKSF for a period of 8 to 18 hours. Lymphocytes and NK cells are then assayed in a standard <sup>51</sup>Cr-release assay for their ability to lyse target cells such as leukemia cell lines, tumor-derived cell lines, or virus-infected fibroblasts. NKSF dramatically increases the ability of NK cells to lyse such target cells at a level comparable to that obtained with interferon alpha and IL-2, well known activators of NK cell cytotoxic activity [See, e.g., G. Trinchieri et al, J. Exp. Med., 147:1314 (1978) and G. Trinchieri et al, J. Exp. Med., 160:1146 (1984)].

In an ADCC assay target cancer cells are coated with antibodies capable of binding to the Fc receptor on NK cells, e.g., IgG<sub>2a</sub>, IgG<sub>3</sub>, and the like. In preliminary assays, the presence of NKSF appears to enhance the killing activity of the NK cells for the coated tumor cells in ADCC. [See, e.g., L. M. Weiner et al, Cancer Res., 48:2568-2573 (1988); F. Hersey et al, Cancer Res., 46:6083-6090 (1988); and C. J. Hansik et al, Proc. Natl. Acad. Sci., 83:7893-97 (1986) for additional information on ADCC.]

Preliminary analysis of NKSF in a B-cell growth factor assay using normal human B cells stimulated with goat anti-human IgM antibody (anti- $\mu$ ) coupled to beads indicates that NKSF may also be characterized by B cell growth factor activity. In this assay the antibody directed against the IgM immunoglobulin on the surface of the B cell activates the B cell and causes it to become responsive to B cell growth factors. [See, C-T K. Tseng et al, J. Immunol., 140:2305-2311 (1988)]. Such antibodies are commercially available.

NKSF was originally detected in the conditioned medium of the human cell line, RPMI 8866, a commercially available cell line [University of Pennsylvania Cell Center] which produces a mixture of lymphokines. This factor may also be produced by other Epstein Barr virus-transformed lymphoblastoid cell lines or from other human cell lines. The purification technique employed in obtaining NKSF from cells which naturally produce it, uses the following steps. These steps include purification through an ion exchange column, e.g., QAE Zeta preparative cartridge [LKB Pharmacea], which indicates that the NKSF protein is anionic. The second purification step is a lentil lectin column which demonstrates that NKSF is, at least in part, a glycoprotein. The eluate from the lentil lectin column

is further purified through a hydroxylapatite column, followed by a heparin sepharose column and a fast protein liquid chromatography (FPLC) Mono-Q column. The NKSF from RPMI 8866 eluted as a single peak in each of the three latter columns. A remaining protein contaminant of about 37 kd is removed by gel filtration chromatography alone or reverse phase HPLC and gel filtration chromatography. The resulting purified homogeneous NKSF was assayed for biological activity in the gamma interferon induction assay of Example 8 and demonstrated a specific activity of greater than  $1 \times 10^7$  dilution units per milligram.

Thus, the homogeneous NKSF may be obtained by applying the above purification procedures, which are described in detail in Example 2 to the conditioned medium of RPMI 8866 or other sources of human NKSF. The RPMI 8866 cell line produces the factor spontaneously, but the level of production can be enhanced by treating the cell line with phorbol esters, such as phorbol dibutyrate. The cells deprived of serum for 48 hours still produce NKSF along with other lymphokines. Procedures for culturing RPMI 8866 (see Example 1) or another cell source of NKSF are known to those of skill in the art.

NKSF or one or both of its subunits or peptide fragments thereof may also be produced via recombinant techniques. To obtain the DNA sequence for cloned NKSF or one or both of its subunits, tryptic digests of the homogeneous polypeptide are prepared. For example, nine tryptic digests found in a subunit of NKSF are identified below:

L-T-I-Q-V  
K-Y-E-N-Y-T  
L-M-D-P-K  
I-W-E-L-K  
V-M-S-Y-L-N-A  
(A)-V S-N-M-L-Q-K  
N-A-S-I-S-V  
T-F-L-R  
D-I-I-K-P-D-P-P-K.

Additionally, the amino terminal sequences of the larger and smaller subunits of NKSF are identified above.

Oligonucleotide probes are synthesized using the genetic code to predict all possible sequences that encode the amino acid sequences of these tryptic digestion products of NKSF. The same procedure may be followed by constructing probes from the above-identified amino terminal sequences of the two subunits of NKSF.

The NKSF gene or the subunit genes can be identified by



using these probes to screen a human genomic library. Alternatively, the mRNA from RPMI 8866 or another cell source of NKSF can be used to make a cDNA library which can be screened with the probes to identify the cDNAs encoding the NKSF polypeptide or the polypeptides of its large and small subunits. Once the cDNAs are identified, they can be co-introduced into any one of a variety of expression vectors to make an expression system for NKSF, or one or both of its subunits.

By such use of recombinant techniques, DNA sequences encoding the NKSF polypeptide or the polypeptides of its large and/or small subunit are obtained which contain DNA sequences encoding one or more of the tryptic fragments or the amino terminal sequences identified above.

One such NKSF clone, named pNK-6, has at least the following DNA and amino acid sequences and codes for all or a portion of the larger NKSF subunit:

16

TABLE I

	20	40	60
5	GAATTCCGTCGACTCTAGAGGCCAGAGCAAGATGTGTCACCAGCAGTTGGTCATCTCTT		
	M C H Q Q L V I S W		
	80	100	120
	GGTTTTCCCTGGTTTTCTGGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATG		
	F S L V F L A S P L V A I W E L K K D V		
10	140		180
	TTTATGTCGTAGAATTGGATTGGTATCCGGAGGCCCTGGAGAAATGGTGGTCCTCACCT		
	Y V V E L D W Y P D A P G E M V V L T C		
15			240
	GTGACACCCCTGAAGAAGATGGTATCACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAG		
	D T P E E D G I T W T L D Q S S E V L G		
			300
20	GCTCTGGCAAACCCCTGACCATCCAAGTCAAAGAGTTTGGAGATGCTGGCCAGTACACCT		
	S G K T L T I Q V K E F G D A G Q Y T C		
			360
25	GTCACAAAGGAGGCGAGGTTCTAAGCCATTCGCTCCTGCTGCTTCACAAAAGGAAGATG		
	H K G G E V L S H S L L L L H K K E D G		
			420
	GAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAACCCAAAAATAAGACCTTTCTAA		
	I W S T D I L K D Q K E P K N K T F L R		

17

480

GATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTGCTGGTGGCTGACGACAATCAGTA  
C E A K N Y S G R F T C W W L T T I S T

540

CTGATTTGACATTCAGTGTCAAAGCAGCAGAGGCTCTTCTGACCCCCAAGGGGTGACGT  
D L T F S V K S S R G S S D P Q G V T C

600

GCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGTATGAGTACT  
G A A T L S A E R V R G D N K E Y E Y S

660

CAGTGGAGTGCCAGGAGGACAGTGCCTGCCAGCTGCTGAGGAGAGTCTGCCCATTGAGG  
V E C Q E D S A C P A A E E S L P I E V

720

TCATGGTGGATGCCGTTCAACAAGCTCAAGTATGAAAACCTACACCAGCAGCTTCTTCATCA  
M V D A V H K L K Y E N Y T S S F F I R

780

GGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATTCTC  
D I I K P D P P K N L Q L K P L K N S R

840

GGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCT  
Q V E V S W E Y P D T W S T P H S Y F S

900

CCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAACCTCTAGAGTCGACG  
L T F C V Q V Q G K S K R E N S R V D G

GAATTC

I

This cloned sequence in plasmid pNK-6 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland on February 3, 1989 under accession number 40545. Additional clones are obtained, and when sequenced, are expected to provide the carboxy-terminal sequence of the larger subunit, and/or the sequence of the smaller subunit of NKSF.

Allelic variations of DNA sequences encoding the peptide sequences and the larger subunit described above are also included in the present invention as well as analogs or derivatives thereof.

Thus the present invention also encompasses these novel DNA sequences, free of association with DNA sequences encoding other primate proteins, and coding on expression for NKSF polypeptides, including those of its large and small subunits. These DNA sequences include those containing one or more of the above-identified DNA and peptide sequences and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences. An example of one such stringent hybridization condition is hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent

hybridization condition is in 50% formamide, 4XSSC at 42°C.

DNA sequences which hybridize to the sequences for NKSF or its subunits under relaxed hybridization conditions and which code on expression for NKSF peptides having NKSF biological properties also encode novel NKSF polypeptides. Examples of such non-stringent hybridization conditions are 4XSSC at 50°C or hybridization with 30-40% formamide at 42°C. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation or disulfide linkages, with the sequences of NKSF and encodes a protein having one or more NKSF biological properties clearly encodes a NKSF polypeptide even if such a DNA sequence would not stringently hybridize to the NKSF sequences.

Similarly, DNA sequences which code for NKSF polypeptides coded for by the sequence of NKSF, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) are also encompassed by this invention. Variations in the DNA sequence of NKSF which are caused by point mutations or by induced modifications to enhance the activity, half-life or

production of the polypeptides encoded thereby are also encompassed in the invention.

NKSF polypeptides may also be produced by known conventional chemical synthesis. Methods for constructing the polypeptides of the present invention by synthetic means are known to those of skill in the art. The synthetically-constructed NKSF polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with NKSF polypeptides may possess NKSF biological properties in common therewith. Thus, they may be employed as biologically active or immunological substitutes for natural, purified NKSF polypeptides in therapeutic and immunological processes.

The NKSF polypeptides provided herein also include factors encoded by sequences similar to those of purified homogeneous and recombinant NKSF protein, or the subunit polypeptides, but into which modifications are naturally provided or deliberately engineered.

Modifications in the peptides or DNA sequences can be made by one skilled in the art using known techniques. Modifications of interest in the NKSF sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenic techniques for such replacement,

insertion or deletion are well known to one skilled in the art. [See, e.g., United States patent 4,518,584.]

Other specific mutations of the sequences of the NKSF polypeptide or the subunit polypeptides described herein may involve modifications of a glycosylation site. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site or at any site of the molecule that is modified by addition of O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

Expression of such altered nucleotide sequences produces variants which are not glycosylated at that site.

Other analogs and derivatives of the sequence of NKSF or of its subunits which would be expected to retain NKSF activity in whole or in part may also be easily made by one of skill in the art given the disclosures herein. One such modification may be the attachment of polyethylene glycol onto existing lysine residues or the insertion of a lysine residue into the sequence by conventional techniques to enable the attachment. Such modifications are believed to be encompassed by this invention.

The present invention also provides a method for producing NKSF polypeptides. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding on expression for an NKSF polypeptide, including a subunit polypeptide, under the control of known regulatory sequences. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent



4,419,446. Coexpression of two different cDNAs simultaneously in CHO cells has been described, for example, in published PCT International Application WO88/08035. Other suitable mammalian cell lines, are the monkey COS-1 cell line, and the CV-1 cell line, originally developed at the Wistar Institute, Philadelphia, Pennsylvania.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

The present invention also provides vectors for use in the method of expression of novel NKSF polypeptides. These vectors contain the novel NKSF DNA

sequences which code for NKSF polypeptides of the invention, including the subunit polypeptides. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of NKSF polypeptides. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

Thus NKSF, purified to homogeneity from cell sources or produced recombinantly or synthetically, may be used in a pharmaceutical preparation or formulation to treat cancer or other disease states which respond to enhanced NK cell activity or increased in vivo production of gamma interferon or GM-CSF. Such pathological states may result from disease, exposure to radiation or drugs, and include for example, leukopenia, bacterial and viral infections, anemia, B cell or T cell deficiencies including immune cell or hematopoietic cell deficiency following a bone marrow transplantation. Therapeutic treatment of cancer and other diseases with these NKSF polypeptide compositions may avoid undesirable side effects caused by treatment with presently available drugs.

It may also be possible to employ one or both of the subunit polypeptides of NKSF or peptide fragments thereof in such pharmaceutical formulations.

The polypeptides of the present invention may also be employed, alone or in combination with other cytokines, hematopoietins, interleukins, growth factors or antibodies in the treatment of cancer or other diseases states. Other uses for these novel polypeptides are in the development of monoclonal and polyclonal antibodies generated by standard methods for diagnostic or therapeutic use.

Therefore, as yet another aspect of the invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of the NKSF protein or a subunit polypeptide or therapeutically effective fragment thereof of the present invention in admixture with a pharmaceutically acceptable carrier. This composition can be systemically administered parenterally. Alternatively, the composition may be administered intravenously. If desirable, the composition may be administered subcutaneously. When systematically administered, the therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable

aqueous solution. The preparation of such a pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

5           The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the  
10           severity of any infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 1-1000 micrograms of NKSF protein or subunit thereof or 50 to 5000 units (i.e., one unit per ml being the concentration of protein which  
15           leads to half maximal stimulation in the gamma interferon induction assay) of protein per kilogram of body weight.

          The therapeutic method and compositions of the present invention may also include co-administration with other human factors. Exemplary cytokines or  
20           hematopoietins for such use include the known factors IL-1, IL-2 and IL-6 particularly. [See, e.g., PCT publications WO85/05124, and WO88/00206; and European patent application 0,188,864.] Other potential candidates for participation in NKSF therapy may also  
25           include IL-4, G-CSF, CSF-1, GM-CSF, IL-3 or

erythropoietin. Growth factors like B cell growth factor, B cell differentiation factor, or eosinophil differentiation factors may also prove useful in co-administration with NKSF.

Similarly, administration of NKSF or a subunit or fragment thereof with or prior to administration of an antibody capable of binding to the Fc receptor on NK cells may enhance ADCC therapy directed against tumors. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

The following examples illustratively describe the purification and characteristics of homogeneous human NKSF and other methods and products of the present invention. These examples are for illustration and do not limit the scope of the present invention.

Example 1. Preparation of Serum-Free RPMI 8866 Cell-Conditioned Medium

The human B-lymphoblastoid cell line RPMI 8866 was maintained in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum (FCS). For preparation of serum free conditioned medium, cells were washed and suspended ( $10^6$  cells/ml) in serum free RPMI 1640 medium containing  $10^{-7}$  M phorbol-12-13-dibutyrate (PdbU) and

cultured for 48 hours at 37°C, 5% CO<sub>2</sub>. The cell free supernatants were harvested by filtration through a 0.2 µm filter [Durapore® hydrophilic cartridge filter, Millipore, Bedford, MA], and Tween-20 and phenylmethylsulfonyl-fluoride (PMSF) were added to 0.02% and 0.1 mM, respectively. The cell conditioned medium was then concentrated 50 fold under pressure using an ultra-filtration cartridge [Spiral-Wound, S1, Amicon, Danvers, MA].

Example 2. Purification of NKSF from Conditioned Medium

The following procedures are presently employed to obtain homogeneous NKSF protein from RPMI 8866 conditioned medium, as described in Example 1 above.

a. Anion Exchange Cartridge Chromatography

Two liters of the crude concentrated conditioned medium was diluted with distilled water to a conductivity of 6m Os/cm and adjusted to pH 8 with 1 M Tris-HCl buffer (pH 8). The concentrate was then applied to five QAE Zetaprep 250 cartridges [Pharmacia] connected in parallel and previously equilibrated with 0.1 M Tris-HCl buffer (pH 8) at a flow rate of 150 ml/min. Unless otherwise cited, all the buffers used for purification contained 0.02% Tween-20 and 0.1 mM PMSF. The cartridges were washed with 3 liters of 0.1 M Tris-HCl buffer (pH 6.8) followed by washing with 1.5 liters of 0.5 M NaCl in

0.1 M Tris-HCl buffer (pH 6.8) and 300 ml fractions were collected. The NKSF activity was eluted with the 0.5 M NaCl-containing wash.

b. Lentil-Lectin Sepharose Chromatography

Pooled NKSF-containing fractions from two separate QAE Zetaprep elutions were pooled and applied directly to a column (2.5x15 cm) of lentil-lectin Sepharose 4B [Pharmacia] which has been equilibrated with 20 mM Tris-HCl buffer (pH 7.2). After washing with five column volumes of equilibration buffer, the column was eluted with three column volumes of 20 mM Tris-HCl buffer (pH 7.2) containing 0.2 M  $\alpha$ -methyl-D-mannopyranoside [Sigma] and 0.5 M NaCl. Approximately half of the NKSF activity was bound by the column and was recovered in the fractions eluted with  $\alpha$ -methyl-D-mannopyranoside.

c. Hydroxylapatite Chromatography

Concentrated material from the pool of NKSF activity which bound to the lentil-lectin Sepharose column was dialyzed against 1 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM  $\text{CaCl}_2$  and 0.15 M NaCl and applied to a Biogel HT [BioRad] column (2x5 cm) previously equilibrated with 1 mM potassium phosphate buffer (pH 6.8) containing 0.1 mM  $\text{CaCl}_2$ . The column was washed with five column volumes of equilibration buffer and eluted with 100 ml of a linear gradient from 1 mM to

400 mM potassium phosphate buffer (pH 6.8) containing 0.15 M NaCl. 4 ml fractions were collected and tested for NKSF activity. A single peak of activity emerged from the column between the approximately 200 mM and 300 mM potassium phosphate.

d. Heparin Sepharose Chromatography

Eluted NKSF-containing fractions from the Biogel HT column were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.2) and applied to a Heparin Sepharose [Pierce, Rockford, IL] column (1x10 cm). The column was washed with five column volumes of 20 mM sodium phosphate buffer (pH 7.2) and eluted with the same buffer containing 1 M NaCl. 3 ml fractions were collected and NKSF activity measured. Essentially all of the activity was bound by the Heparin column and recovered in the 1 M NaCl wash.

e. Mono Q Chromatography

Pooled fractions from the Heparin Sepharose column were dialyzed against 20 mM Tris-HCl buffer (pH 6.8) containing 1% ethylene glycol and 0.1 mM PMSF but no Tween-20 (buffer A) and concentrated to 2 ml using a stirred cell [Amicon] with a YM 10 membrane. The sample was applied to a Mono Q (5/5) column [Pharmacia-FPLC apparatus] and eluted with a linear gradient from 0 M to 1 M NaCl in buffer A (pH 6.8). 0.5 ml fractions



were collected and tested for NKSF activity. The activity emerged from the column as a single peak between approximately 220 mM and 270 mM NaCl.

f. Gel Filtration Chromatography

Pooled fractions containing NKSF activity from the Mono Q column were concentrated to 100 microliters by Speedvac Concentrator [Savant, Farmingdale, NY] and applied to a FPLC Superose 12 column. Chromatography was run with 50 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl, 1% ethylene glycol and 0.1 mM PMSF. Flow rate was 0.6 ml/minute and 0.5 ml fractions were collected. NKSF protein (70 kd) was separated from the approximately 37 kd protein contaminant.

Alternatively, the pooled Mono-Q fractions may be subjected to reverse-phase HPLC (C8 column) prior to the step (f) described above, to separate the protein contaminant from the active 70 kd protein.

Example 3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli [Laemmli, U. K., Nature, 227:680-685 (1970)] on 10% acrylamide slab gels (0.75 mm thickness). After electrophoresis the gels were either stained by the silver-nitrate method using a silver staining reagents

[BioRad] or cut into 2 mm slices and eluted in 0.5 ml RPMI medium for 4 hours at 24°C and assayed for NKSF activity. Apparent molecular weight was determined with protein standards, phospholipase b (94 kd), bovine serum albumin (67 kd), ovalbumin (43 kd), carbonic anhydrase (30 kd), soybean trypsin inhibitor (20 kd) and lactalbumin (14.4 kd).

SDS-PAGE analysis (non-reducing conditions) of the Mono Q column fractions (Example 2, step (e)) beginning with several fractions which eluted before the NKSF activity, continuing right through the active fractions and ending with fractions which eluted after the peak of NKSF activity, revealed that the presence of two proteins (70 kd and 37 kd) correlated with the presence of NKSF activity in the various Mono Q fractions. The active fractions were rerun on a second non-reducing gel and the proteins were eluted from the regions corresponding to the 70 kd and 37 kd bands and tested for NKSF activity. The activity all correlated with the 70 kd species indicating that this protein is NKSF.

The 70 kd species was eluted from the gel, iodinated using chloramine T [Sigma, St. Louis, MO] and rerun on a second SDS gel after boiling for two minutes in the presence of the reducing agent,  $\beta$ -mercaptoethanol

(10%). Under these conditions, the 70 kd species resolved into two distinct subunits of molecular weights 40kd and 30kd, indicating that the native NKSF may be a disulfide-bonded heterodimer of these subunit polypeptides. Alternatively, NKSF may be a dimer formed by multiples of the larger or smaller subunits. The reduction of the native 70kd NKSF appeared to destroy all of its ability to induce peripheral blood lymphocyte production of gamma interferon.

#### Example 4. Recovery of Protein

Starting with 500 liters of RPMI 8866 cell-free conditioned medium, the final pooled active fractions from the Mono Q column contained approximately 10  $\mu$ g of protein, estimated from the intensities of silver staining by control proteins analyzed in parallel on the same gel. Approximately 6  $\mu$ g of this corresponded to the 70 kd NKSF protein. The estimated specific activity of the 70 kd NKSF is  $1 \times 10^7$  u/mg. The overall recovery of NKSF activity in the preparation was 2%.

#### Example 5. NKSF Protein Composition

Homogeneous NKSF is reduced as described in the SDS-PAGE example above and digested with trypsin. Alternatively, non-reduced NKSF may be obtained from a reverse-phase HPLC column and digested with trypsin.

Nine tryptic fragments are isolated having the following amino acid sequences with tentatively identified amino acids in parentheses:

Fragment 1 - L-T-I-Q-V

Fragment 2 - L-M-D-P-K

Fragment 3 - K-Y-E-N-Y-T

Fragment 4 - I-W-E-L-K

Fragment 5 - V-M-S-Y-L-N-A

Fragment 6 - (A)-V-S-N-M-L-Q-K

Fragment 7 - N-A-S-I-S-V

Fragment 8 - T-F-L-R

Fragment 9 - D-I-I-K-P-D-P-P-K.

Additionally, the amino acid sequences of the amino termini of each subunit of NKSF were determined from the isolated 40 kd and 30kd species of NKSF after reduction, as described in Example 3. The amino terminal sequence from the 40kd subunit was as follows:

I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-G-E-M.

The amino terminal sequence above as well as Fragments 1, 3, 4, 8 and 9 proved to be derived from the amino acid sequence of the clone of larger subunit identified in Table I above.

The amino terminal sequence from the 30kd smaller subunit was as follows, with tentatively

identified amino acids enclosed in parentheses: (X)-N-L-P-V-A-(P)-P-D-P-(S)-M-F-P. The (X) indicates that the first residue in this sequence could not be determined.

Probes consisting of pools of oligonucleotides or unique oligonucleotides are designed according to the method of R. Lathe, J. Mol. Biol., 183(1):1-12 (1985). The oligonucleotide probes are synthesized on an automated DNA synthesizer.

Because the genetic code is degenerate (more than one codon can code for the same amino acid) a mixture of oligonucleotides must be synthesized that contains all possible nucleotide sequences encoding the amino acid sequence of the tryptic fragment. It may be possible in some cases to reduce the number of oligonucleotides in the probe mixture based on codon usage because some codons are rarely used in eukaryotic genes, and because of the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [see J. J. Toole et al, Nature, 312:342-347 (1984)]. The regions of the amino acid sequences used for probe design are chosen by avoiding highly degenerate codons where possible. The oligonucleotides are synthesized on an automated DNA synthesizer and the probes are then radioactively labelled with polynucleotide kinase and  $^{32}\text{P}$ -ATP.

cDNA is then synthesized from polyadenylated RNA from the RPMI 8866 cell line and cloned into lambda ZAP [Stratagene Cloning Systems, La Jolla, CA] or other suitable vectors using established techniques (see Toole et al cited above). Recombinants from this library are plated and duplicate nitrocellulose replicas made of the plates. The oligonucleotides are kinased with  $^{32}\text{P}$  gamma ATP and hybridized to the replicas at a temperature predicted from the length and base composition of the probes [See, J. Singer-Sam et al, Proc. Nat'l. Acad. Sci. USA, 80:802-806 (1983) and S.V. Suggs et al, in "Developmental Biology Using Purified Genes", ICN-UCLA Symposium on Molecular and Cellular Biology, eds. Brown D.D. and Fox, C.F. (Academic, NY), vol. 23, pp. 683-693 (1981)] in standard hybridization solution overnight. The filters are then washed in 0.5XSSC at the same temperature until the background radioactivity is lowered to an acceptable level to allow autoradiography. Alternatively, the hybridization and washes may be performed in the presence of tetraalkylammonium salt solution [See K. A. Jacobs et al, Nucl. Acids Res., 16:4637-4650 (1988).] Duplicate positives are plaque purified. Clones containing all or a portion of the nucleotide sequence necessary to encode human NKSF are obtained. One of the clones obtained according to this

procedure is described above and deposited as ATCC

Example 6. Expression of Recombinant Human NKSF

To produce NKSF, the cDNAs encoding its subunits are transferred into appropriate expression vectors, of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression, by standard molecular biology techniques.

One such vector for mammalian cells is pXM [Y. C. Yang et al, Cell, 47:3-10 (1986)]. This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells [See, e.g., Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985)]. The pXM vector is linearized with the endonuclease enzyme XhoI and subsequently ligated in equimolar amount separately to the cDNA encoding the NKSF subunits that were previously modified by addition of synthetic oligonucleotides [Collaborative Research, Lexington, MA] that generate Xho I complementary ends to generate constructs for expression of each subunit of NKSF. If the two polypeptides derive from two different

mRNAs, two different cDNAs must be expressed simultaneously in the same host or independently in different hosts and the subunits purified separately. The final active NKSF is assembled by renaturation of the individual subunits.

If two subunits of NKSF are derived from a single mRNA, i.e., they are generated by proteolytic cleavage of a single precursor polypeptide, the vector is ligated in equimolar amount to the individual cDNA in a similar manner as described above. The corresponding cDNA can be expressed in various hosts with appropriate vectors..

a. Mammalian Cell Expression

To obtain expression of the NKSF protein for use in the assays described below, the pXM constructs containing the cDNAs for the individual subunits (or the single cDNA encoding both subunits if they derive from a single precursor) are mixed and transfected into COS cells, for example. The conditioned medium from the transfected COS cells contains NKSF biological activity as measured in the gamma interferon induction assay.

The mammalian cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers,



promoters, and the like, may be obtained from natural sources or synthesized by known procedures. See, Kaufman et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman, Proc. Natl. Acad. Sci., USA, 82:689-693 (1985).

5 Exemplary mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable.

10 Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting. For stable integration of the vector DNAs, and for subsequent amplification of the integrated vector DNAs, both by conventional methods, CHO cells may  
15 be employed. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al, Cell, 36:391-401 (1984)] and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other suitable mammalian cell lines include but  
20 are not limited to, HeLa, COS-1 monkey cells, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Because the NKSF protein is a dimer, the two subunits, if different, e.g. a larger and a smaller  
25 subunit, must be expressed simultaneously in the same

host or separately and refolded together to yield active NKSF. If, however, the NKSF subunits are generated by cleavage of a precursor encoded by a single cDNA, then, at least in mammalian cells containing the appropriate protease, the single cDNA can be expressed to yield functional NKSF.

Where the two subunits require simultaneous expression in mammalian cells, the two cDNAs must be introduced into the cells using two different selectable genes or markers. As discussed below in Example 7, this can readily be achieved in CHO cells using the dihydrofolate reductase (DHFR) gene as one marker and adenosine deaminase (ADA) as the other marker. Any combination of two genes which can be independently selected in any mammalian cell line are useful for this purpose. For example, a CHO cell line is independently developed for expression of one subunit under ADA selection and a different cell line is developed for expression of the other subunit under DHFR selection. The cell lines are fused in polyethylene glycol under double selection to yield stable lines expressing both subunits. Alternatively, the DNAs are introduced simultaneously or sequentially into the same cells, thereby yielding lines expressing active NKSF.

Stable transformants are then screened for expression of the product by standard immunological, biological or enzymatic assays. The presence of the DNA and mRNA encoding the NKSF polypeptides may be detected by standard procedures such as Southern blotting and RNA blotting. Transient expression of the DNA encoding the polypeptides during the several days after introduction of the expression vector DNA into suitable host cells, such as COS-1 monkey cells, is measured without selection by activity or immunologic assay of the proteins in the culture medium.

One skilled in the art can also construct other mammalian expression vectors comparable to the pXM vector by, e.g., inserting the DNA sequences of the NKSF subunits from the respective plasmids with appropriate enzymes and employing well-known recombinant genetic engineering techniques and other known vectors, such as pJL3 and pJL4 [Gough et al., EMBO J., 4:645-653 (1985)] and pMT2 (starting with pMT2-VWF, ATCC #67122; see PCT application PCT/US87/00033). The transformation of these vectors with both NKSF subunits into appropriate host cells can result in expression of the NKSF polypeptides.

b. Bacterial Expression Systems

Similarly, one skilled in the art could manipulate the sequences encoding the NKSF subunits by

eliminating any mammalian regulatory sequences flanking the coding sequences and inserting bacterial regulatory sequences to create bacterial vectors for intracellular or extracellular expression of the NKSF subunits of the invention by bacterial cells. The DNA encoding the NKSF polypeptides may be further modified to contain different codons to optimize bacterial expression as is known in the art. Preferably the sequences encoding the mature NKSF subunits are operatively linked in-frame to nucleotide sequences encoding a secretory leader polypeptide permitting bacterial expression, secretion and processing of the mature NKSF polypeptides, also by methods known in the art. The simultaneous expression of both subunits of NKSF in E. coli using such secretion systems is expected to result in the secretion of the active heterodimer. This approach has yielded active chimeric antibody fragments [See, e.g., Bitter et al, Science, 240:1041-1043 (1988)].

Alternatively, the individual subunits are expressed in the mature form separately from the two different cDNAs in E. coli using vectors for intracellular expression and the subunits are isolated separately, mixed and refolded by procedures well known in the art. See, for example, U.S. Patent 4,512,922.

The compounds expressed through either route in bacterial host cells may then be recovered, purified, and/or characterized with respect to physicochemical, biochemical and/or clinical parameters, all by known methods.

c. Insect or Yeast Cell Expression

Similar manipulations can be performed for the construction of an insect vector for expression of NKSF polypeptides in insect cells [See, e.g., procedures described in published European patent application 155,476]. If the NKSF subunits are derived from a single cDNA, this cDNA will be expressed in insect cells. Alternatively, if the NKSF subunits are derived from two different cDNAs, each subunit is separately inserted into an insect cell vector and the two resulting vectors co-introduced into insect cells to express biologically active NKSF.

Similarly yeast vectors are constructed employing yeast regulatory sequences to express either the individual NKSF subunits simultaneously, or, if the protein is derived from a single precursor, the cDNA encoding that precursor, in yeast cells to yield secreted extracellular active NKSF heterodimer. Alternatively the individual subunits may be expressed intracellularly in yeast, the individual polypeptides isolated and finally,

refolded together to yield active NKSF. [See, e.g., procedures described in published PCT application WO 86/00639 and European patent application EP 123,289.]

Example 7. Construction of CHO Cell Lines Expressing High Levels of NKSF

One method for producing high levels of the NKSF protein of the invention from mammalian cells involves the construction of cells containing multiple copies of the two cDNAs encoding the individual NKSF subunits or of the cDNA encoding the NKSF precursor if the subunits derive from a single polypeptide.

In the latter case, the single cDNA is co-transfected with an amplifiable marker, e.g., the DHFR gene for which cells containing increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., (1982) supra. This approach can be employed with a number of different cell types.

For example, the pXM vector containing the NKSF precursor gene in operative association with other plasmid sequences enabling expression thereof (Example 6) is introduced into DHFR-deficient CHO cells, DUKX-BII, along with a DHFR expression plasmid such as pAdd26SVpA3 [Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985)] by calcium phosphate coprecipitation and transfection.

DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum. Transformants are checked for expression of NKSF by bioassay, immunoassay or RNA blotting and positive pools are subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol. Cell Biol., 5:1750 (1983). The amplified lines are cloned, and NKSF protein expression is monitored by the gamma interferon induction assay. NKSF expression is expected to increase with increasing levels of MTX resistance.

If the two NKSF polypeptides are each derived from separate mRNAs, each corresponding cDNA is expressed simultaneously in CHO cells. Two different selectable markers, e.g., DHFR and ADA, may be employed. One of the cDNAs is expressed using the DHFR system using, e.g., the vector pXM to express one of the NKSF subunits and pAdd26SVpA3 to express DHFR as described above for a single precursor NKSF protein. The second subunit is also expressed using the vector pXM but the marker is obtained through co-transfection with the plasmid pSV2ADA [Kaufman et al, Proc. Natl. Acad. Sci. USA, 83:3136 (1986)], which directs expression of ADA in mammalian cells. The pXM vector construct containing the second

subunit is transfected into DHFR-deficient CHO DUKX-BII cells along with pSV2ADA. The transfected cells are selected for growth in increasing concentrations of 2'-deoxycoformycin (dCF) beginning with 0.01 micromolar with subsequent step-wise increments up to 40 micromolar. The expression of the individual cDNAs (one subunit under DHFR selection in one cell line and the other subunit under ADA selection in a second cell line) is performed through a combination of mRNA blotting to test for transcription and immunoanalysis to test for protein production. The cells which express one of the subunits under ADA selection and the cells which express the other subunit under DHFR selection are finally fused in polyethylene glycol using methods well established in the art to yield a single cell line, resistant to both dCF and MTX and expressing both subunits to yield biologically active NKSF.

A cell line expressing either subunit under either drug selection may be generated. Secondly the cDNA expressing the other subunit may be introduced under the second drug selection to yield cells expressing both subunits simultaneously. (See, e.g., published PCT International Application WO88/08035 for an exemplary description of independently amplifying a first gene



linked to a DHFR gene and a second gene linked to an ADA gene.)

Additionally the two pXM constructs expressing both NKSF subunits may be mixed with the plasmid for expression of DHFR and the plasmid expressing ADA. The combined selection of both drugs may be used and transformants tested for NKSF activity directly to obtain cell lines expressing the heterodimer.

In any of the expression systems described above, the resulting cell lines can be further amplified by appropriate drug selection, resulting cell lines recloned and the level of expression assessed using the gamma interferon induction assay described herein.

#### Example 8. Biological Activities of Human NKSF

The following assays were performed using either the homogeneous NKSF described in Example 2 or a partially purified version of NKSF. The recombinant version of the molecule is expected to exhibit NKSF biological properties in these same assays or other assays.

When fresh human peripheral blood mononuclear cells (PBMC) or phytohemagglutinin (PHA)-induced blasts are cultured with NKSF, significant amounts of gamma interferon are detected in the supernatant. Moreover, NKSF synergizes with IL-2, phorbol dibutyrate (PdBu), and

PHA in inducing gamma interferon production. Northern blot analyses show that NKSF, alone or in combination with other factors, induces accumulation of gamma interferon mRNA. Gamma interferon message was found in both purified T and NK populations. Preincubation with the protein synthesis inhibitor, cycloheximide (CHX), leads to a superinduction of gamma interferon mRNA following stimulation with NKSF. HLA-DR(+) accessory cells are required for gamma interferon production by T and NK cells. Induction of gamma interferon mRNA can be detected as early as 1 hour after treatment with NKSF of PHA blasts. The details of the assay are described below.

a. Gamma Interferon Induction Assay

NKSF activity was measured by the induction of gamma interferon (gamma-IFN) expression in cultures of human peripheral blood lymphocytes (PBLs). In the assay, 100  $\mu$ l of human PBLs suspended ( $10^7$  cells/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS was added to 100  $\mu$ l of sample to be tested in a microtiter plate [U-bottom, 96-well, Costar, Cambridge, MA] and incubated for 18 hours at 37°C, 5% CO<sub>2</sub>. Samples to be tested included purified NKSF, dialyzed cell free supernatant from 48 hour phorbol diester stimulated RPMI 8866 cells, and recombinant IL-2

[Genetics Institute, Inc., PCT application WO85/05124]. After incubation, 100  $\mu$ l of cell free supernatant was withdrawn from each well and the level of gamma-IFN produced measured by radioimmunoassay [Centocor Gamma Interferon Radioimmunoassay, Centocor, Malvern, PA]. One unit of NKSF per ml is the concentration required to produce one-half of the maximal gamma-IFN produced in the presence of optimal concentrations of NKSF.

There was a linear positive correlation between the amount of gamma-IFN produced in each well to the amount of NKSF in culture.

In addition to gamma-IFN, NKSF induces T and NK cells to produce GM-CSF and tumor necrosis factor. The assay of production of these cytokines is performed as above and the supernatant is assayed for the presence of the cytokines by specific biological assays or by radioimmunoassays [Cuturi et al, J. Exp. Med., 165:1581-1594 (1987)]. Alternatively, the induction of the cytokine genes is measured by evaluating the accumulation of mRNA transcripts of the three cytokines in the lymphocytes treated with NKSF. Lymphocytes are cultured for 4 to 18 hours with NKSF, RNA is extracted by established methods, fractionated by agarose gel electrophoresis, blotted on nitrocellulose, and hybridized with <sup>32</sup>P-labeled cDNA probes for the IFN-gamma,

GM-CSF, or tumor necrosis factor genes (Northern blotting). Extent of hybridization is determined by autoradiography and densitometry.

5 NKSF induces production of IFN-gamma and TNF from purified human NK cells. When assayed as described under the gamma interferon induction assay of part (a) above, NK cells are able to lyse various target cells by two mechanisms. One mechanism is spontaneous lysis, in the absence of specific sensitization, of a variety of  
10 target cells, including leukemia- and solid tumor-derived cell lines, virus-infected cells, and, in some cases, normal cell lines. The second mechanism is ADCC. Preliminary evidence indicates that NKSF may enhance the ability of NK cells to lyse more efficiently target cells  
15 coated with IgG antibodies with an Fc portion able to bind to the NK cell Fc receptor.

b. NK Assay

In order to assay for the enhancement of NK cell spontaneous cytotoxicity by NKSF, PBLs or  
20 purified NK cells ( $5 \times 10^6$  cells/ml) are incubated for 18 hours in RPMI 1640 medium, 10% heat inactivated FCS, in the presence of various dilutions of NKSF. PBLs are then washed and added, at PBL-target cells ratio from 1:1 to 100:1, to  $10^4$   $^{51}\text{Cr}$ -labeled target cells in a U-bottomed  
25 microtiter plate (final volume 200  $\mu\text{l}$ ). After 4 hours,

the plates are centrifuged, the cell-free supernatant is collected and lysis of target cells is evaluated by the release of the  $^{51}\text{Cr}$ -label from the cells. NKSF increases several-fold the cytotoxicity of NK cells when assayed against the following target cells: malignant hematopoietic cell lines (i.e. K562, Daudi, U937, HL-60, ML3, Molt 4, Jurkat, THP-1), solid tumor-derived cell line (rhabdomyosarcoma, melanoma), and normal foreskin-derived fibroblast strains. The enhancement of NK cell-mediated cytotoxicity by NKSF is not secondary to the production of IFN-gamma, tumor necrosis factor, or IL-2, produced by the PBL treated with NKSF. The cytotoxic assay, the methods for NK cell purification, and for the quantitative evaluation of enhancement of NK cell-mediated enhancement by cytokines are described in detail in G. Trinchieri et al, J. Exp. Med., 147:1314 (1978); G. Trinchieri et al, J. Exp. Med., 160:1147 (1984); and B. Perussia et al, Natural Immunity and Cell Growth Regulation, 6:171-188 (1987).

c. ADCC Assay

In a standard antibody dependent cell mediated cytotoxicity assay, preliminary results show that partially purified NKSF of the present invention enhanced NK cell killing of antibody coated tumor target cells in a dose dependent manner. For antibodies capable of

binding to the Fc receptor of the NK cell, the ADCC response of NK cells was enhanced by the addition of NKSF.

d. Co-mitogenic effect of NKSF

PBLs ( $0.5 \times 10^6$ /ml) are cultured in 200  $\mu$ l of RPMI 1640 medium supplemented with 10% heat inactivated human AB serum. After 3 and 6 days the PBLs are pulsed for 6 hours with  $^3\text{H}$ -thymidine and DNA synthesis (proliferation) is evaluated by the  $^3\text{H}$ -thymidine uptake in the cells by collecting the cells on glass filters using a Skatron cell harvester and counting the cell-associated  $^3\text{H}$ -Thymidine by liquid scintillation using a Packard Tricarb beta counter. NKSF has minimal effect on PBL proliferation by itself, but is strongly co-mitogenic with phytohemagglutinin (PHA-M Welcome, 1:100) at day 6 of culture and with phorbol diesters (TPA or PDBu,  $10^{-8}$  or  $10^{-7}\text{M}$ , respectively) at both day 3 and day 6. Cell cycle analysis is performed by flow cytofluorometry (Cytofluorograf 50H, Ortho Diagnostics) using a technique combining DNA staining with immunofluorescence staining according to London et al, J. Immunol., 137:3845 (1986). This analysis has shown that the PBLs affected by the co-mitogenic effect of NKSF are T cells either CD4 or CD8 positive.

e. GM-CSF Induction Assay

Induction of GM-CSF expression in cultures of human PBLs was measured. In the assay, 100  $\mu$ l of human PBLs suspended ( $10^7$  cells/ml) in RPMI 1640 culture medium supplemented with 10% heat-inactivated FCS was added to 100  $\mu$ l of sample to be tested in a microtiter plate [U-bottom, 96-well, Costar, Cambridge, MA] and incubated for 13 hours at 37°C, 5% CO<sub>2</sub>. After incubation, 100  $\mu$ l of cell-free supernatant was withdrawn from each well and the level of GM-CSF produced measured by enzyme-linked immunosorbent assay (ELISA) using two murine monoclonal antibodies against human GM-CSF (3/8.20.5 and 2/3.1, supplied by Genetics Institute, Inc.) recognizing different epitopes. Using recombinant human GM-CSF (Genetics Institute, Inc.) as a standard, the detection limit of this assay was 50 pg/ml.

Numerous modifications and variations in practice of this invention are expected to occur to those skilled in the art.

CLAIMS

1. Natural killer cell stimulatory factor protein substantially free from association with other proteinaceous materials.
2. The protein according to claim 1 comprising the same or substantially the same DNA and amino acid sequences of Table I or fragments thereof.
3. The protein according to claim 1 comprising a subunit having an apparent molecular weight of approximately 40kd and having the same or substantially the same amino terminal sequence as follows: I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-G-E-M.
4. The protein according to claim 1 comprising a subunit having an apparent molecular weight of approximately 30-35 kd having the same or substantially the same amino terminal sequence as follows: X-N-L-P-V-A-(P or X)-P-D-P-(S or X)-M-F-P, wherein X is any amino acid.



5. The protein according to claim 1 characterized chemically as comprising one or more of the following sequences of amino acids:

L-T-I-Q-V

K-Y-E-N-Y-T

L-M-D-P-K

I-W-E-L-K

V-M-S-Y-L-N-A

(A)-V-S-N-M-L-Q-K

N-A-S-I-S-V

T-F-L-R

D-I-I-K-P-D-P-P-K

I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-

G-E-M,

and

X-N-L-P-V-A-(P or X)-P-D-P-(S or X)-M-F-P,

wherein X is any amino acid.

6. The protein according to claim 1 characterized biologically by a specific activity in a gamma interferon induction assay of greater than  $1 \times 10^7$  dilution units/mg.

7. The protein according to claim 1 having one or more of the following characteristics:

(1) an apparent molecular weight under non-reducing conditions on SDS PAGE of approximately 70-80 kd;

(2) a subunit having an apparent molecular weight under reducing conditions on SDS PAGE of approximately 40 kd;

(3) a subunit having an apparent molecular weight under reducing conditions on SDS PAGE of approximately 30-35 kd;

(4) an isoelectric point of 4.3 on isoelectric focusing gel;

(5) an isoelectric point of 4.8 on isoelectric focusing gel;

(6) elution from hydroxylapatite column as a single peak;

(7) elution from heparin-sepharose column as a single peak;

(8) elution from an FPLC Mono-Q column as a single peak;

(9) biological activity in a gamma IFN inducing assay with PBLs;

(10) biological activity in a GM-CSF inducing assay with PBLs;

(11) biological activity in activating NK cells to kill leukemia and tumor-derived cells;

(12) biological activity in a tumor necrosis factor induction assay using PHA-activated T lymphocytes;

(13) co-mitogenic activity on peripheral blood T lymphocytes.

8. The protein according to claim 1 produced by subjecting conditioned medium from RPMI 8866 to sequential purification through a QAE Zeta Prep cartridge, a lentil lectin column, a hydroxylapatite column, a heparin sepharose column and a fast protein liquid chromatography Mono-Q column, wherein said NKSF elutes from the latter column as a single peak.

9. The protein according to claim 1 produced by culturing a cell line transformed with a cDNA sequence encoding expression of NKSF in operative association with an expression control sequence therefor.

10. The protein according to claim 3 wherein said subunit comprises the same or substantially the same amino terminal sequence as follows: M-C-H-Q-Q-L-V-I-S-W-F-S-L-V-F-L-A-S-P-L-V-A-I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-

P-D-A-P-G-E-M-V-V-L-T-C-D-T-P-E-E-D-G-I-T-W-T-L-D-Q-S-S-  
E-V-L-G-S-G-K-T-L-T-I-Q-V-K-E-F-G-D-A-G-Q-Y-T-C-H-K-G-G-  
E-V-L-S-H-S-L-L-L-L-H-K-K-E-D-G-I-W-S-T-D-I-L-K-D-Q-K-E-  
P-K-N-K-T-F-L-R-C-E-A-K-N-Y-S-G-R-F-T-C-W-W-L-T-T-I-S-T-  
D-L-T-F-S-V-K-S-S-R-G-S-S-D-P-Q-G-V-T-C-G-A-A-T-L-S-A-E-  
R-V-R-G-D-N-K-E-Y-E-Y-S-V-E-C-Q-E-D-S-A-C-P-A-A-E-E-S-L-  
P-I-E-V-M-V-D-A-V-H-K-L-K-Y-E-N-Y-T-S-S-F-F-I-R-D-I-I-K-  
P-D-P-P-K-N-L-Q-L-K-P-L-K-N-S-R-Q-V-E-V-S-W-E-Y-P-D-T-W-  
S-T-P-H-S-Y-F-S-L-T-F-C-V-Q-V-Q-G-K-S-K-R-E-N-S-R-V-D-G-  
I, or fragments thereof.

11. A process for preparing homogeneous NKSF comprising subjecting conditioned medium from RPMI 8866 to sequential purification through a QAE Zeta Prep cartridge, a lentil lectin column, a hydroxylapatite column, a heparin sepharose column and a fast protein liquid chromatography Mono-Q column, wherein said NKSF elutes from the latter column as a single peak.

12. The process according to claim 11 further comprising subjecting the Mono-Q column eluate to gel filtration chromatography.

13. The process according to claim 11 optionally including a reverse phase HPLC purification before said gel filtration chromatography.

14. A process for producing NKSF or a subunit thereof comprising culturing a cell line transformed with a cDNA sequence encoding expression of NKSF or a subunit thereof in operative association with an expression control sequence therefor.

15. A DNA sequence coding for NKSF or a subunit thereof comprising a sequence of nucleotide bases encoding one or more of the following amino acid sequences:

L-T-I-Q-V

K-Y-E-N-Y-T

L-M-D-P-K

I-W-E-L-K

V-M-S-Y-L-N-A

(A)-V-S-N-M-L-Q-K

N-A-S-I-S-V

T-F-L-R

D-I-I-K-P-D-P-P-K

60

I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-

G-E-M,

and

X-N-L-P-V-A-(P or X)-P-D-P-(S or X or T)-M-F-P,

wherein X is any amino acid.

16. The DNA sequence according to claim 15 comprising the same or substantially the same sequence of nucleotide bases or fragments thereof:

20	40	60
GAATTCCGTCGACTCTAGAGGCCAGAGCAAGATGTGTCACCAGCAGTTGGTCATCTCTT		
80	100	120
GGTTTCCCTGGTTTTTCTGGCATCTCCCCTCGTGGCCATATGGGAACTGAAGAAAGATG		
140		180
TTTATGTCGTAGAATTGGATTGGTATCCGGAGGCCCTGGAGAAATGGTGGTCCTCACCT		
		240
GTGACACCCCTGAAGAAGATGGTATCACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAG		
		300
GCTCTGGCAAAACCCTGACCATCCAAGTCAAAGAGTTTGGAGATGCTGGCCAGTACACCT		
		360
GTCACAAAGGAGGCGAGGTTCTAAGCCATTCGCTCCTGCTGCTTCACAAAAGGAAGATG		
		420
GAATTTGGTCCACTCATATTTTAAAGGACCAGAAAGAACCCAAAATAAGACCTTTCTAA		

61

480  
GATGCGAGGCCAAGAATTATTCTGGACGTTTCACCTGCTGGTGGCTGACGACAATCAGTA  
540  
CTGATTTGACATTCAGTGTCAAAGCAGCAGAGGCTCTTCTGACCCCCAAGGGGTGACGT  
600  
GCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGTATGAGTACT  
660  
CAGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTGAGGAGAGTCTGCCCATTTGAGG  
720  
TCATGGTGGATGCCGTTTACAAGCTCAAGTATGAAACTACACCAGCAGCTTCTTCATCA  
780  
GGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAAGCCATTAAAGAATTCTC  
840  
GGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCT  
900  
CCCTGACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAACCTCTAGAGTCGACG  
GAATTC

17. The DNA sequence according to claim 15 comprising a sequence of nucleotide bases encoding the same or substantially the same amino acid sequence or fragments thereof: M-C-H-Q-Q-L-V-I-S-W-F-S-L-V-F-L-A-S-P-L-V-A-I-W-E-L-K-K-D-V-Y-V-V-E-L-D-W-Y-P-D-A-P-G-E-M-V-V-L-T-C-D-

T-P-E-E-D-G-I-T-W-T-L-D-Q-S-S-E-V-L-G-S-G-K-T-L-T-I-Q-V-  
K-E-F-G-D-A-G-Q-Y-T-C-H-K-G-G-E-V-L-S-H-S-L-L-L-L-H-K-K-  
E-D-G-I-W-S-T-D-I-L-K-D-Q-K-E-P-K-N-K-T-F-L-R-C-E-A-K-N-  
Y-S-G-R-F-T-C-W-W-L-T-T-I-S-T-D-L-T-F-S-V-K-S-S-R-G-S-S-  
D-P-Q-G-V-T-C-G-A-A-T-L-S-A-E-R-V-R-G-D-N-K-E-Y-E-Y-S-V-  
E-C-Q-E-D-S-A-C-P-A-A-E-E-S-L-P-I-E-V-M-V-D-A-V-H-K-L-K-  
Y-E-N-Y-T-S-S-F-F-I-R-D-I-I-K-P-D-P-P-K-N-L-Q-L-K-P-L-K-  
N-S-R-Q-V-E-V-S-W-E-Y-P-D-T-W-S-T-P-H-S-Y-F-S-L-T-F-C-V-  
Q-V-Q-G-K-S-K-R-E-N-S-R-V-D-G-I.

18. A cell transformed with a DNA sequence of claim 15 in operative association with an expression control sequence.

19. The cell according to claim 18 comprising a mammalian or bacterial cell.

20. Homogeneous NKSF having a specific activity in the gamma interferon induction assay of greater than  $1 \times 10^7$  dilution units per mg polypeptide.

21. A pharmaceutical composition comprising a therapeutically effective amount of NKSF or a subunit thereof in a pharmaceutically effective vehicle.



22. The composition according to claim 21 further comprising therapeutically effective amounts of an additional cytokine, hematopoietin, or growth factor.

23. The composition according to claim 21 where said cytokine is selected from the group consisting of IL-1, IL-2 and IL-6.

24. A plasmid vector comprising the DNA sequence of claim 15.

25. A method for treating cancer comprising administering to a patient an effective amount of NKSF or a subunit thereof.

26. The method according to claim 25 further comprising administering simultaneously or sequentially with said NKSF an effective amount of at least one hematopoietin, cytokine, growth factor or antibody capable of binding to the Fc portion of NK cells.

27. The method of claim 25 wherein said hematopoietin is IL-1, IL-2 or IL-6.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 89/05027

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) \*

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/06, C 07 K 7/10, C 12 N 15/26, A 61 K 37/02

## II. FIELDS SEARCHED

Minimum Documentation Searched \*

Classification System:

Classification Symbols

IPC5: C 07 K; C 12 N; C 12 P; C 07 G; A 61 K

Documentation Searched other than Minimum Documentation  
to the extent that such Documents are included in the Fields Searched \*

## III. DOCUMENTS CONSIDERED TO BE RELEVANT \*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
A	US, A, 4473555 (NESTOR, JR. ET AL) 25 September 1984, see column 1, line 46 - line 52; column 2, line 17 - line 23  --	1-27
A	Journal of Biological Response Modifiers, Vol. 4, 1985, James E. Talmadge: "Immunoregulation and Immunostimulation of Murine Lymphocytes by Recombinant Human Interleukin-2", see page 18 - page 34 see page 19, line 18-28  --	1-27
A	PROGRESS IN IMMUNOLOGY V, Vol. 5, 1983, Gy.G. Petranyi et al: "Natural Killer Cells in Man: Genetic and Other Factors Regulating Their Activity", see page 1169; page 1174 - page 1175, page 1178-page 1181, chapter IV page 1174-1178  --	1-27

\* Special categories of cited documents: \*\*

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"Z" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

13th March 1990

Date of Mailing of this International Search Report

- 5 APR 1990

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

T.K. WILLIS

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	SCIENCE, Vol. 214, 1981, Ronald B. Herberman et al: "Natural Killer Cells: Their Role in Defenses Against Disease ", see page 24 - page 30 page 26 2 col  --	1-27
P,X	Dialog Information Services, File 154, Medline 82-90. Dialog accession no. 89361252, Kobayashi M et al: "Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes", J Exp Med, Sep 1 1989, 170 (3) p.827-45  -- -----	1-27

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US-A- 4473555	25/09/84	AU-D- 3428784	26/04/85
		EP-A- 0138616	24/04/85
		JP-A- 60087299	16/05/85

CROSS REFERENCE OF INDEX A OF INTERNATIONAL  
APPLICATIONS WHERE PUBLICATION UNDER ARTICLE  
21 PCT TAKES THE PLACE OF THE PUBLICATION  
OF THE EUROPEAN PATENT APPLICATION.  
(ARTICLE 158 PARAGRAPH 1 EPO)

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